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(54) Title: METHODS TO DETECT AND TREAT DISEASES CAUSED BY BACTERIAL ALLERGENS

(57) Abstract

The invention provides materials and methods useful in the diagnosis and treatment of *H.pylori* induced gastric disease. Included are polypeptides containing one ore more epitopes immunologically identifiable with epitopes of *H.pylori* proteins. These polypeptides are useful in assays which measure IgE in biological samples from putatively infected individuals. They are also useful for immunotherapy of infected individuals.

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METHODS TO DETECT AND TREAT DISEASES CAUSED BY BACTERIAL ALLERGENS

Technical Field

This invention is related to the field of allergens, more specifically to the identification of bacterial allergens, and to their use in disease diagnosis and treatment.

Background of the Invention

A number of idiopathic recurrent diseases are of unknown etiology. Some of these diseases are thought to be linked to infection by a microorganism. However, the causal relationship between the microorganism and the disease is often not established. The twin disorders of chronic gastritis and peptic ulcer disease are within this category.

Chronic gastritis and peptic ulcer disease are diseases of major significance. Five to ten percent of all individuals develop chronic gastritis and or gastroduodenal ulcers in their lifetime. Ulcer disease is a common cause of morbidity. The annual prevalence of symptomatic peptic ulcer disease in the United States of America is approximately 18 per 1,000 adults (or 4,500,000 people). Approximately 350,000 new cases of peptic ulcer disease are diagnosed each year.

Diagnosis of these diseases is usually performed by gastroduodenal endoscopy, an invasive and costly procedure. Treatment encompasses oral medication, dietary controls, and surgery. Rarely is it definitive, and these chronic conditions often have cycles of improvement and relapse.

Since the report by Marshall (Lancet 1983, i:1273) that the bacteria Helicobacter pylori is

physically associated with the lesions of chronic gastritis, a great deal of work has been done in an effort to elucidate a causal relationship between the organism and the chronic disease. Early speculations regarding localized pH changes induced by H. pylori, the release of toxins (Hupertz et al. (1988), Eur J. Clin Microbiol Infect Dis 7:576), and destructve enzymes (Slomiany et al. (1989), Am J. Gastroenterol 84:1273), and the differences between different strains of the bacteria (Eaton et al (1989), Infect Immun (U.S.) 57:1119) have not resulted in firm conclusions concerning the etiology of the disease. Moreover, the search for a reasonable explanation of cause and effect has been further complicated by the recognition that a significant number of clinically well subjects also carry the organism.

Description of Related Art

Caska et al. (1972), Radioimmunosorbent Assay of Allergens (J. Allergy and Clin. Immunol. 49:1), describes a Radioallergosorbent (RAST) test to detect IgE directed to specific allergens.

Nalebuff et al. (1979), The Study of IgE in the Diagnosis of Allergic Disorders in an Otolarygology Practice (Otolaryngol Head Neck Surg. 87:351), describes a modified RAST test.

Lambert et al. (1978), Diffuse Varioliform Gastritis (Digestion 17:159), reportedly provide studies showing that the lesions of chronic gastritis contain IgE positive plasma cells, which the authors interpret as suggesting an allergic origin for this disease.

Andre et al. (1983), Evidence for Anaphylactic Reactions in Peptic Ulcer and Varioliform Gastritis (Annals of Allergy 51:325). The average numbers and

class distribution of IgE-containing cells in patients with various types of gastritis/ulcers as compared healthy subjects reportedly were examined. The authors interpret the results as confirmatory for the theory that mucosal anaphylaxis may be the cause of the gastric lesions.

Calenoff et al. (1983), Bacteria-Specific IgE in Patients with Nasal Polyposis (Arch Otolaryngol 109: 372). The authors describe the use of a modified RAST test to detect IgE specific to bacterial antigens in patients exhibiting chronic nasal polyposis.

Warren (1983), Unidentified Curved Bacilli on Gastric Epithelium in Active Chronic Gastritis (Lancet, 1273). The author reports the observation that small curved and S-shaped bacilli were observed in 135 gastric biopsy specimens. The bacilli were most frequently correlated with inflammation, and were almost always present in acute chronic gastritis.

Peterson (1991), Helicobacter pylori and Peptic Ulcer Disease (New England J. Med. 174:1043), is a review article on the association between H. pylori (formerly called Campylobacter pilori) and gastritis/ulcer disease.

Disclosure of the Invention

Summary

Using a modified RAST test, it was discovered that there was a high positive correlation between gastritis/ulcer disease and the presence of IgE directed to specific subfractions of protein allergens of H. pylori. These results indicate that an adverse immune reaction to these bacteria is responsible for the

pathological reaction, in particular, the existence of a hypersensitivity reaction mediated by specific IgE.

In the modified RAST test purified protein allergens were linked to a solid support. Prior to reaction with the protein allergens of H. pylori, the serum to be tested was treated to remove IgA and IgG. This "scrubbing" step was essential for the detection of the allergen-specific IgE.

The identification of protein allergens of H. pylori associated with gastritis/ulcer disease allows for a relatively non-invasive detection of the disease by a modified RAST test. It also allows for treatment of the disease by immunotherapy, using purified protein allergens.

Accordingly, one aspect of the invention is a method of measuring IgE which binds immunologically to a bacterial allergen. Serum suspected of containing the IgE is reacted with an extract of the bacteria coupled to a solid support, followed by washing and reacting with labelled anti-IgE, and detecting labeled anti-IgE bound to the solid support, the improvement comprising reacting the serum with a composition capable of removing IgA and IgG from the serum prior to the reacting with the bacterial extract coupled to the solid support, wherein the amount of composition used is sufficient to remove IgA and IgG which interferes with the IgE binding to the bacterial allergen.

Another aspect of the invention is a method of preparing purified protein allergen from bacteria comprising: (a) treating bacteria containing a protein allergen with acetone to remove lipid components; (b) disrupting the acetone-treated bacteria in a solution comprised of buffer, salt, metal chelator, protease inhibitor, and benazamidine; (c) separating a protein

containing fraction from complex carbohydrates and nucleic acids; (d) collecting a composition comprised of proteins which are of molecular weight at least about 1,000; and (e) separating the proteins of the composition of (d) by ion-exchange chromatography.

Still another aspect of the invention an immunotherapeutic method of treating an individual for a disease resulting from an allergic reaction to a bacterial infection comprising introducing into the individual a composition consisting essentially of protein allergens from the bacteria, wherein the conditions of the introduction are sufficient alleviate the symptoms of the allergic reaction.

Another aspect of the invention is a method of determining whether an individual has an allergic response to Helicobacter pylori, the method comprising: (a) providing serum from an individual suspected of containing IgE to H. pylori allergens; (b) providing a composition consisting essentially of H. pylori protein allergens; (c) reacting the serum of (a) with the composition of (b) under conditions which allow immunological binding between IgE and an allergen to which it is directed; and (d)detecting IgE-allergen complexes formed, if any, between IgE in the serum of (a) and a protein allergen in the composition of (b).

Still another aspect of the invention is a composition consisting essentially of protein allergens of H. pylori.

Another aspect of the invention is a protein allergen of H. pylori coupled to a solid substrate.

Yet another aspect of the invention is a method of treating an individual for H. pylori induced gastritis comprising introducing into the individual a composition comprised of a polypeptide which contains one or more

epitopes that are immunologically identifiable with immunogenic epitopes of H. pylori, wherein the polypeptide is in an amount sufficient to relieve an allergic reaction to H. pylori in the individual, and wherein the composition is further comprised of a suitable excipient.

Still another aspect of the invention is a diagnostic kit comprised of a polypeptide containing at least one epitope which is immunologically identifiable with an H. pylori epitope, packaged in a suitable container, and a means for detecting immunological complexes formed between the polypeptide and IgE in the biological sample, if any.

Yet another aspect of the invention is a composition comprised of a structural analog of an epitope of an H. pylori allergen, wherein the structural analog binds to an IgE paratope.

Another aspect of the invention is a composition comprised of a purified polyclonal antibody directed to a polypeptide allergen of H. pylori.

Yet another aspect of the invention is a composition comprised of a monoclonal antibody directed to a polypeptide allergen of H. pylori.

Brief Description of the Figures

Figure 1 is a graph showing the effect of scrubbing serum with Protein A on the detection of anti-H pylori IgE in a modified RAST test.

Figure 2A is a graph showing the serum IgE levels of IgE directed to subfractions of H. pylori protein allergens in healthy individuals (controls).

Figure 2B is a graph showing the serum IgE levels of IgE directed to subfractions of H. pylori protein allergens in gastritis patients.

Figure 3 is a plot of the net total IgE immunological reactivity of serum from control and gastritis patients using all available H. pylori protein fractions isolated from an HPLC DEAE column.

Figure 4 is a plot of the net total IgE immunological reactivity of serum from control and gastritis patients with the proteins in fractions 59, 64, 66, 68, 72 and 74 of the HPLC DEAE column.

Modes for Carrying Out the Invention

The present invention stems from the discovery that individuals with chronic gastritis or gastroduodenal ulcers have serum IgE specific for protein allergens of H. pylori, implicating hypersensitivity to this microorganism in the etiology of the diseases.

H. pylori is most likely an innocuous colonizer of the gastric mucosa. It dwells just beneath the protective mucous layer and probably feeds from it without much harm to the host or to the host's protective defenses against the gastric acid. The inflammatory process recognized in chronic gastrtitis results in those individuals who possess the genetic proclivity toward allergy and then have the necessary MHC II antigen framework for presenting the H. pylori allergenic proteins as allergens. A qualitative and/or quantitative reduction in the secretion of protective mucus by the goblet cells probably occurs thus making the underlying mucosa vulnerable. In addition, a likely increase in local histamine production may take place in response to the allergic reaction and is absorbed into the vascular plexus of the stomach thus leading to an increase in gastric acid production. These two phenomena may together result in increased irritation of the early gastric lesions and, along with the constant allergic

reaction to H. pylori, lead to lesion enlargement and chronicity.

Based upon the discovery discussed above, it is possible to design immunoassays to detect an H. pylori induced allergic reaction in individuals. In one aspect, these immunoassays utilize purified protein allergens, and are preferable to endoscopy since they may be performed in vitro and are relatively non-invasive. In addition, the discovery allows for a novel treatment of these diseases; i.e., immunotherapy with compositions comprised of at least one purified protein allergen of H. pylori, and/or with an allergoid of a protein allergen of H. pylori.

The practice of the present invention will employ, unless otherwise indicated, convantional techniques of protein purification, microbiology, molecular biology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature.

As used herein, the term "allergen" refers to an antigen that gives rise to allergic sensitization by IgE antibodies.

The term "allergoid" refers to a chemically modified allergen that gives rise to antibody of the IgG but not IgE class, thereby reducing allergic symptoms.

The term "individual", as used herein, refers to a vertebrate, particularly members of the mammalian species, and includes but is not limited to domestic animals, sports animals, and primates, including humans.

The term "allergy", as used herein, denotes an altered state of immune reactivity, usually denoting hypersensitivity.

As used herein, "Immunologically identifiable with/as" refers to the presence of epitope(s) and

polypeptides(s) which are also present in the designated polypeptide(s). Immunological identity may be determined by antibody binding and/or competition in binding; these techniques are known to those of average skill in the art, and are also illustrated infra.

As used herein, "epitope" refers to an antigenic determinant of a polypeptide. An epitope could comprise 3 amino acids in a spatial conformation which is unique to the epitope. Generally an epitope consists of at least 5 such amino acids, and more usually, consists of at least 8-10 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance.

A polypeptide is "immunoreactive" when it is "immunologically reactive" with an antibody, i.e., when it binds to an antibody due to antibody recognition of a specific epitope contained within the polypeptide. Immunological reactivity may be determined by antibody binding, more particularly by the kinetics of antibody binding, and/or by competition in binding using as competitor(s) a known polypeptide(s) containing an epitope against which the antibody is directed. The techniques for determining whether a polypeptide is immunologically reactive with an antibody are known in the art. An "immunoreactive" polypeptide may also be "immunogenic". As used herein, the term "immunogenic polypeptide" is a polypeptide that elicits a cellular and/or humoral immune response, whether alone or linked to a carrier in the presence or absence of an adjuvant.

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of at least one antibody combining site. An "antibody

combining site" or "binding domain", is formed from the folding of variable domains of an antibody molecule(s) to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an epitope of an antigen, which allows an immunological reaction with the antigen. An antibody combining site may be formed from a heavy and/or a light chain domain (VH and VL, respectively), which form hypervariable loops which contribute to antigen binding. A "paratope" is an antibody combining site for an epitope, the simplest form of an antigenic determinant. The term "antibody" includes, for example, vertebrate antibodies, hybrid antibodies, chimeric antibodies, altered antibodies, univalent antibodies, the Fab proteins, and single domain antibodies.

The term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. The term "polypeptide" does not connote the method by which the molecule was made, and thus includes naturally occurring molecules, as well as molecules made by chemical or recombinant synthesis.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual,

including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of in vitro cell culture constituents.

The term "coupled" as used herein refers to attachment by covalent bonds or by strong non-covalent interactions (e.g., hydrophobic interactions, hydrogen bonds, etc.). Covalent bonds may be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like.

The term "support" refers to any solid or semi-solid surface to which a desired polypeptide. Suitable supports include glass, plastic, metal, polymer gels, and the like, and may take the form of beads, wells, dipsticks, membranes, and the like.

The term "label" as used herein refers to any atom or moiety which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a polynucleotide or polypeptide.

The term "treatment" as used herein, refers to prophylaxis and/or therapy.

The term "immunogenic" refers to an agent used to stimulate the immune system of a living organism, so that one or more functions of the immune system are increased and directed towards the immunogenic agent.

In one embodiment of the invention, an individual's allergic sensitivity to H. pylori is determined by detecting IgE specific to H. pylori allergens. Any method of detecting IgE specific for an allergen known in the art may be used.

For example, in one method, one or more polypeptides comprised of epitopes immunologically identifiable with epitopes of allergens (a term which

includes allergen polypeptides) are coupled to a solid substrate. A biological sample suspected of containing IgE specific for allergens from the material is reacted with the allergen-substrate complex, and IgE that reacted immunologically with the allergen of the complex is detected. An example of this kind of assay is the Radioallergosorbent (RAST) test.

Generally, in the RAST test an allergen extract is coupled to cellulose particles or paper discs. Patient's serum containing IgE antibody or a standard serum is reacted with the allergen-coupled immunosorbent. After thorough washing, labeled anti-IgE is reacted with the immunosorbent. After further washing, the label on the separated sorbent is determined and is a measure of the amount of specific serum IgE antibodies to that allergen. In a preferred mode, the RAST test is modified to increase its sensitivity by removing IgG and/or IgA antibodies which may interfere with IgE binding to the allergen. This is particularly critical when measuring serum IgE specific to H. pylori allergens. Reactants capable of remvoving IgG and/or IgA are known in the art, and include, for example, Protein G, anti-human IgG and anti-human IgA, as well as Protein A. For convenience, these reactants may be affixed to a solid substrate, including, for example, Sepharose. The amount of the reactants used is sufficient to removing interfering IgG and IgA, but not the IgE which is to be detected. The determination of the desired amount is by methods known to those of skill in the art.

A method of removing interfering IgG and/or IgA antibodies by incubation of the serum with Protein A is discussed in the Examples, infra. Generally, the amount of Protein A which is used is sufficient to prevent the

blocking antibodies from competing with the IgE having the same specificity.

The modified RAST test may also include the use of purified protein allergens. Methods of purifying proteins are known in the art, and include, for example, differential extraction, salt fractionation, chromatography on ion exchange resins, affinity chromatography, centrifugation, and the like. See, for example, Methods in Enzymology for a variety of methods for purifying proteins. An example of a purification procedure which separates protein allergens of H. pylori from carbohydrates, lipids, and nucleic acids is presented in the Examples. Further separation of the protein allergens by HPLC chromatography on DEAE identified subfractions of protein allergens from H. pylori that bind to IgE from individuals with chronic gastritis and/or gastroduodenal ulcers; IgEs specific for these allergans were essentially absent in normal control individuals. Allergens from these fractions would be especially useful in immunoassays.

For convenience, polypeptides comprised of one or more epitopes which are immunologically identifiable with epitopes of H. pylori allergens may be packaged in diagnostic kits. Diagnostic kits include the polypeptides in suitable containers and a means for detecting immunological complexes formed between the polypeptide and IgE in the biological sample, if any. In some cases, the polypeptides may be affixed to a solid substrate. The kit may also contain other suitably packaged reagents and materials needed for the particular diagnostic protocol, for example, standards, buffers, as well as instructions for conducting the test using the kit ingredients.

In another embodiment of the invention, individuals suspected of having a propensity for, or suffering from H. pylori induced gastric disease are treated with substances which reduce the allergic response to the microorganism. Treatment may be with, for example, a composition containing purified protein allergens, or with recombinant polypeptides or anti-idiotype antibodies which are immunologically identifiable with the protein allergen by virtue of one or more immunogenic epitopes which are immunologically cross-reactive with those on H. pylori protein allergen. One or more allergens contained within DEAE fractions 59, 64, 66, 68, 72 and 74, the preparation of which is described in Example 1, may be particularly suitable.

Treatment may also be with, for example, allergoids of H. pylori protein allergens. Methods of preparing allergoids from antigens are known in the art. Typically, mild formalin or glutaraldehyde treatment of the antigen reduces the allergenicity (IgE formation) without affecting the antigenicity (IgG "blocking" antibody formation).

Treatment may also be with, for example, compositions containing at least one structural analog of an epitope of a protein allergen, which binds to the corresponding IgE paratope. Structural analogs are organic molecules which are capable of assuming the appropriate charge distribution and hydrophobic/hydrophilic characteristics to allow binding to the paratope in a fashion which mimics the immunologic binding of the epitope.

When the goal is alleviation of the allergic reaction by immunotherapy in the form of hyposensitization, the treated individual receives injections of a composition comprised of one or more

relevant allergens continuously. Treatment is begun at a dosage low enough to avoid any local or systemic reactions, and frequent injections, usually once or twice a week are administered at increasing dosages until the highest dose the patient can tolerate without excessive local or systemic reactions is reached. This is a maintenance dose, which is then continued at less frequent intervals, usually every 2-6 weeks depending upon the individual's response. However, the actual dosage and treatment regimen will depend upon the individual treated, and will be determined by the person administering the treatment.

In another embodiment of the invention, the immunoreactive polypeptides (including allergens) or structural analogs of epitopes, are prepared into vaccines. Vaccines may be prepared from one or more immunogenic polypeptides. If recombinant, these polypeptides may be expressed in a variety of host cells (e.g., bacteria, yeast, insect, or mammalian cells), or alternatively may be isolated from the bacterial preparations.

The preparation of vaccines which contain an immunogenic polypeptide(s) or structural analogs of epitopes as active ingredients, is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like

and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an H. pilori immunoreactive sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium

saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

The proteins may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of about 5 micrograms to about 250 micrograms of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reenforce the immune

response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In another embodiment of the invention, a polypeptide containing one or more epitopes immunologically identifiable with epitopes of an H. pylori allergen are used to prepare antibodies to H. pylori epitopes, using the polypeptide as an immunizing agent, and methods known to those of skill in the art. The antibodies prepared may be purified polyclonal antibodies, single-chain antibodies, monoclonal antibodies, antibody fragments, and the like. These antibodies may be used, for example, for purification by affinity chromatography polypeptides of interest. More specifically, they can be used to purify polypeptides containing epitopes immunologically identifiable with epitopes of H. pylori allergens, including the allergens themselves.

In turn, antibodies to H. pylori epitopes may be used for the preparation of anti-idiotype antibodies. These anti-idiotype antibodies are comprised of a region which mimics the epitope of the allergen. Anti-idiotype may be synthesized using methods known in the art, and will usually use antibodies directed to H. pylori epitopes as an immunizing agent.

Anti-idiotype antibodies may be useful in immunotherapy of individuals sensitive to H. pylori allergens, as well as for the purification of and/or detection of antibodies directed to H. pylori antigens containing epitopes which immunologically cross-react with the anti-idiotype antibodies.

The immunogenic polypeptides prepared as described above are used to produce antibodies, including polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an immunogenic polypeptide bearing an H. pylori epitope(s). Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an H. pylori epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker (1987) IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London). Alternatively, polyclonal antibodies may be isolated from an individual previously infected with H. pylori, and purified by the methods discussed above.

Monoclonal antibodies directed against H. pylori epitopes can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al. (1980) HYBRIDOMA TECHNIQUES PRINCIPLES AND PRACTICE, SECOND EDITION (Springer-Verlag, N.Y.).; Hammerling et al. (1981) MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS; Kennett et al. (1980) MONOCLONAL ANTIBODIES; see also, U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against H. pylori epitopes can be

screened for various properties; i.e., for isotype, epitope affinity, etc.

Antibodies, both monoclonal and polyclonal, which are directed against H. pylori epitopes are particularly useful in diagnosis, and those which are neutralizing are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies.

Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the antigen of the infectious agent against which protection is desired. See, for example, Nisonoff, A., et al. (1981), Clin. Immunol. Immunopathol. 21:397-406; and Dreesman et al. (1985), J. Infect. Disease 151:761. Techniques for raising anti-idiotype antibodies are known in the art. See, for example, Grych (1985), Nature 316:74; MacNamara et al. (1984), Science 226:1325; and Uytdehaag et al. (1985), J. Immunol. 134:1225. These anti-idiotype antibodies may also be useful for treatment, vaccination and/or diagnosis of H. pylori induced gastritis and/or gastroduodenal ulcers, as well as for an elucidation of the immunogenic regions of H. pylori antigens

EXAMPLES

Described below are examples of the present invention which are provided for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

Example 1

Isolation of H. pylori Protein Allergens and Covalent Coupling of the Allergens to Paper Discs

Processing of H. pylori

Four grams, wet weight, of H. pylori (ATCC strain 43504; ATCC, Bethesda, MD, USA) were cultured essentially by the method of Smibert. Smibert, Ann. Rev. Microbiol. 1978 32:67. More specifically, H. pylori obtained from the American Type Culture Collection, ATCC No. 43504, was removed aspectically from its vial, suspended in 1 ml sterile Difco Brucella broth, and transferred by an in inoculating loop to 3 separate Brucella Agar plates (Anaerobe systems, San Jose, CA). The plates were incubated at 35 deg C for 5 days in a microaerophilic atmosphere of 85% N_2, 10% CO_2, and 5% O_2. After incubation the plates were removed and examined. Tiny grayish-white colonies were observed. Microscopic examination of a Gram-stained smear showed large oxbow-shaped and loops of Gram-negative rods (5 microns), which are typical of H. pylori.

H. pylori in colonies from the 5 day plates were transferred to a fresh set of Brucella plates, and the plates were incubated microaerophilically at 35 deg C for 3 to 5 days. After 3 days a more luxuriant growth of H. pylori colonies occurred. These colonies were used as the inoculum for a broth seed culture.

A broth seed culture was prepared by transferring to several 10 ml screw-capped tubes 5 ml sterile Brucella broth with 5% horse serum (GIBCO BRL), and colonies collected by swab from the plates. All tubes were incubated at 35 deg C under a microaerophilic atmosphere for 3 to 5 days. If a heavy degree of turbidity was observed in the tubes after this period, the culture was examined for purity by microscopic examination of a Gram stained slide.

The broth seed culture was used as an inoculum for one liter of sterile Difco Brucella broth containing 5% horse serum. The inoculated culture was grown in a 3 liter flask by incubation at 35 deg C in a microaerophilic atmosphere for 3 to 5 days. When a moderate degree of turbidity was observed, the culture was checked for purity as described above. One liter of culture generally yielded an unwashed cell amount of about 2.0 grams.

In order to isolate the protein allergens, the living organisms from the liter culture were pelleted by centrifugation at 3,000 RPM, 4 deg C for 15 minutes. They were attenuated and grossly defatted by suspension and vortexing in ice cold acetone for 15 minutes. The attenuated bacteria was then repelleted by similar centrifugation. The pellet was resuspended in 20 ml of cold buffer containing 50 mM sodium phosphate, pH 7.3, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 micrograms/ml PMSF and 100 micrograms/ml of benzamidine. Ten mL or 150-210 micron, acid washed glass beads (Sigma, St. Louis, MO, USA) was added and the suspension sonicated at setting No. 7 using a 400 Watt Branson Sonifier II ultransonic cell disruptor with a regular tip. The suspension was thus sonicated for 15 minutes while being cooled in a methanol ice bath. The resulting mixture was then centrifuged as above and the supernatant saved.

Gradient Centrifugation

The supernatant was centrifuged for 1 hr at 100,000 g. 4 deg C, in a Beckman SW 40Ti rotor (Beckman, Palo Alto, CA, USA). To the resulting supernatant was added 0.456 gm/ml of RbCl (Aldrich Chemical Co., Milwaukee, Wis., USA). The solution was then centrifuged at 4 deg C for 48 hrs. in a Beckman 70 Ti rotor (the

first 24 hrs at 65,000 RPM and the second 24 hrs at 48,000 RPM). The supernatant contents of each gradient tube were collected in ten equal fractions beginning at the bottom of each tube. The pellet in each tube representing most of the residual complex carbohydrates and nucleic acids containing in the pregradient supernatant was discarded.

Ion Exchange Chromatography

Each gradient fraction was dialyzed against 20 mM sodium phosphate buffer, pH 7.0, at 4 deg C using dialysis tubing with a 1,000 MW cutoff. An approximation the protein content per fraction was made by spectrophotometry at a wavelength of 280 nm. Ninety percent of the detected protein was found in fractions 2 through 6, inclusive; these fractions were pooled. The pooled fractions were then loaded onto a Bio-Sil DEAE analytical anion exchange HPLC column (BioRad, Richmond, CA, USA) and a 30 minute linear gradient run achieving 100 per cent Buffer B at the end of the gradient. The equilibrating buffer (Buffer A) was 20 mM Sodium phosphate, pH 7.0. The salt containing buffer (Buffer B) was 20 mM sodium phosphate, pH 7.0, with 1.0 M NaCl. The eluted fractions were collected and the protein of each quantified as before. The flow-through (void) fraction containing macromolecules and cationic molecules was loaded onto a Bio-Sil SP cation exchange column (BioRad) and run under the exact gradient conditions as for the DEAE run. The resulting eluted fractions were also quantified for protein.

Covalent Coupling of H. pylori Proteins to Paper Discs

CnBr activated paper discs were made essentially by the method of Ceska. Ceska et al., J.

Allergy and Clin. Immun. 49:1 (1972). More specifically, paper discs (diameter 6 mm) were cut with a punch from Schleicher and Schuell 589 red ribbon filter paper. The discs were allowed to swell for 30 minutes in water. CNBr solution (5 per cent in water), was added and mixed with a mechanical stirrer for 3 minutes in a water bath at 19 deg C. NaOH (1 M), was added dropwise to maintain the pH in the range of 10.0 to 10.5. The suspension was immediately poured into about a ten-fold excess of cold $NaHCO_3$ solution (5 mM, 4 deg C). After thorough mixing, the solution was decanted. The wash with $NaHCO_3$ solution was repeated eleven times. The paper discs then were washed twice each with 500 ml of 25%, 50%, and 75% acetone in a graded series, followed by washing four times with 500 ml acetone (reagent grade, 4 deg C). They were then placed on a filter paper under hood ventilation for 3 hours for drying, packaged with dessicant pouches in plastic bags, and stored at -20 deg C until use.

A sufficient volume was taken from each of the elution samples collected during the ion exchange runs and diluted with 50 mM sodium carbonate buffer, pH 9.6, to yield a 3 ml solution containing 300 micrograms of protein. To each was added 30 CNBr activated paper discs and the mixture was then placed under gentle agitation for 48 hrs at 4 deg C in order to covalently couple the various proteins to their respective discs. The protein discs were washed and blocked with ethanolamine as described by Ceska, supra.

Example 2

A Modified RAST Procedure for Detecting IgE Specific to H. pylori Allergens

IgE specific for H. pylori allergens was assayed for using a modified RAST procedure. Part of the procedure was essentially as described by Nalebuff et al. (Nalebuff et al., Otolarygol Head Neck Surg 89:271 (1981).). More specifically, an aliquot of 100 microliters of serum was incubated overnight with an appropriate allergen disc and washed three times with 50 mM phosphate buffered saline (PBS), pH 7.3, containing 0.1% Tween 20. This was followed by a second overnight incubation with I^{125}-labelled anti-IgE specific for the De-2 determinant. After being washed and prior to being counted, the allergen discs were placed into fresh tubes in a gamma counter for the amount of time previously selected by a time control. The time control consists of 25 units of WHO-standardization IgE that is run against a PRIST anti-IgE disc for the time needed for the IgE to bind 25,000 counts. This time is used in the counting of all subsequent tests.

Background levels for individual patients were determined by running each protein A scrubbed serum (see below) against 4 blank discs, and calculating a median value representing the individual's background. Values twice this background level or greater were deemed positive. Determining the individual background level for each patient increases the precision of the assay, since it takes into account the variability corresponding directly to total serum IgE (not just that specific for the bacterial allergens).

As shown in Figure 1, in order to detect H. pylori IgE, it was essential to scrub the serum samples to remove most IgG and IgA antibodies before incubation with discs containing H. pylori protein allergens.

Scrubbing was by incubation with recombinant Protein A/Sepharose (Zymed, S. San Francisco, CA, USA).

More specifically, two ml of serum per one ml of Protein A/Sepharose were incubated with agitation for 1 hr. The suspension was then centrifuged at 1500 RPM for 15 min. and the serum supernatants collected.

The results in Figure 1 were obtained by taking two aliquots of the same serum from a patient with document gastritis and H. pylori colonization, and subjecting one of these aliquots to the scrubbing procedure. The scrubbed and unscrubbed samples from equivalent amounts of serum were then subjected to the remainder of the RAST procedure using discs containing H. pylori protein allergens, as described above. In the figure, the serum IgE levels detected in the scrubbed (open squares) and unscrubbed samples (closed circles) are compared. As seen from the graph, the scrubbed samples allowed the binding of IgE to the H. pylori protein allergens which had eluted from the DEAE column with a peak at fraction number 66. This binding was not detected in the unscrubbed sample. A repeated assay yielded similar results.

Example 3

Analysis of Patient Sera for H. pylori Specific IgE

Ten consecutive gastritis/GI ulcer patients that were disease positive by endoscopy, two patients without lesions by endoscopy, and 12 apparently asymptomatic control patients were tested using the modified RAST procedure with scrubbing, as described in Example 2.

All ten disease positive patients had measurable quantities of H. pylori specific IgE in their sera. The two normal endoscopy patients were IgE negative, and six of twelve asymptomatic control subjects

were also IgE positive to some of the HPLC eluted proteins. As shown in Figure 2, each IgE positive patient appeared to react to differently to the various HPLC fractionated proteins.

The prevalence of IgE positive reactivity toward the individual chromatographed fractions for each positive patient in the "asymptomatic" and "gastritis" patients was examined. There were several H. pylori protein fractions to which the disease group patients reacted with greater exclusivity then the "asymptomatic" patients. This more exclusive reactivity was with DEAE fractions 59, 64, 66, 68, 72 and 74.

Figure 3 shows a plot of the net total IgE immunological reactivity of serum from control and gastritis patients using all available H. pylori protein fractions isolated from an HPLC DEAE column. Figure 4 is a plot of the net total IgE immunological reactivity of serum from control and gastritis patients with the proteins in fractions 59, 62, 65, 70, 64, 68, 71, 73, and 74.

Industrial Significance

Polypeptides containing one or more epitopes immunologically identifiable with epitopes of H. pylori proteins (including but not limited to purified allergens, recombinantly or synthetically produced polypeptides, and allergoids) are useful in the diagnosis of H. pylori induced gastric diseases, and may also be useful for treatment of these diseases. These polypeptides are also useful for the production of antibodies, both purified polyclonal and monoclonal, directed towards epitopes of H. pylori. The antibodies, in their turn, are useful in the purification of polypeptides containing one or more epitopes immunologically identifiable with epitopes of H. pylori proteins. Monoclonal antibodies, in particular, are useful in the production of anti-idiotype antibodies, which in turn, are useful for the detection of antibodies containing specific epitopes of H. pylori, and may also be useful in the production of vaccines for H. pylori induced diseases.

The methods described herein use one or more polypeptides containing one or more epitopes of H. pylori, and detect IgE directed to H. pylori allergens. These methods, particularly the modified RAST method, are useful for the diagnosis of H. pylori induced gastric diseases.

CLAIMS

1. In a method of measuring IgE which binds immunologically to a bacterial allergen, wherein serum suspected of containing the IgE is reacted with an extract of the bacteria coupled to a solid support, followed by washing and reacting with labelled anti-IgE, and detecting labeled anti-IgE bound to the solid support, the improvement comprising reacting the serum with a composition capable of removing IgA and IgG from the serum prior to the reacting with the bacterial extract coupled to the solid support, wherein the amount of composition used is sufficient to remove IgA and IgG which interferes with the IgE binding to the bacterial allergen.

2. The method of claim 1, further comprising using purified protein allergen coupled to the solid support.

3. The method of claim 1, wherein the composition is comprised of Protein A.

4. The method of claim 2, wherein the composition is comprised of Protein A.

5. A method of preparing purified protein allergen from bacteria comprising:

(a) treating bacteria containing a protein allergen with acetone to remove lipid components;

(b) disrupting the acetone-treated bacteria in a solution

comprised of buffer, salt, metal chelator, protease inhibitor, and benzamidine;

(c) separating a protein containing fraction from complex carbohydrates and nucleic acids;

(d) collecting a composition comprised of proteins which are of molecular weight at least about 1,000;

(e) separating the proteins of the composition of (d) by ion-exchange chromatography.

6. The method of claim 5, wherein the ion-exchange chromatography is on an anion exchange high pressure liquid chromatography (HPLC) column.

7. The method of claim 2, wherein the purified protein allergen is prepared by the method of claim 5.

8. The method of claim 2, wherein the purified protein allergen is prepared by the method of claim 6.

9. A composition comprised of a protein allergen prepared by the method of claim 5.

10. A composition comprised of a protein allergen prepared by the method of claim 6.

11. An immunotherapeutic method of treating an individual for a disease resulting from an allergic

reaction to a bacterial infection comprising introducing into the individual a composition consisting essentially of protein allergens from the bacteria, wherein the conditions of the introduction are sufficient alleviate the symptoms of the allergic reaction.

12. A method of determining whether an individual has an allergic response to Helicobacter pylori, the method comprising:

(a) providing serum from an individual suspected of containing IgE to H. pylori allergens,

(b) providing a composition consisting essentially of H. pylori protein allergens;

(c) reacting the serum of (a) with the composition of (b) under conditions which allow immunological binding between IgE and an allergen to which it is directed; and

(d) detecting IgE-allergen complexes formed, if any, between IgE in the serum of (a) and a protein allergen in the composition of (b).

13. The method of claim 12, wherein the serum provided has been reacted with a composition capable of removing IgA and IgG from the serum in amount sufficient to remove IgG and IgA which interferes with formation of the IgE-allergen complex.

14. The method of claim 13, wherein the composition is comprised of Protein A.

15. A composition consisting essentially of protein allergens of H. pylori.

16. A protein allergen of H. pylori coupled to a solid substrate.

17. The composition of claim 15, wherein the the protein allergens of H. pylori are prepared according to the method of claim 5.

18. The composition of claim 15, wherein the purified protein allergens of H. pylori are prepared according to the method of claim 6.

19. A method of treating an individual for H. pylori induced gastritis comprising introducing into the individual a composition comprised of a polypeptide which contains one or more epitopes that are immunologically identifiable with immunogenic epitopes of H. pylori, wherein the polypeptide is in an amount sufficient to relieve an allergic reaction to H. pylori in the individual, and wherein the composition is further comprised of a suitable excipient.

20. The method of claim 19, wherein the composition is comprised of a purified protein allergen of H. pylori.

21. The method of claim 20, wherein the composition is comprised of an allergoid of an H. pylori protein allergen.

22. A diagnostic kit comprised of a polypeptide containing at least one epitope which is

immunologically identifiable with an H. pylori epitope, packaged in a suitable container, and a means for detecting immunological complexes formed between the polypeptide and IgE in the biological sample, if any.

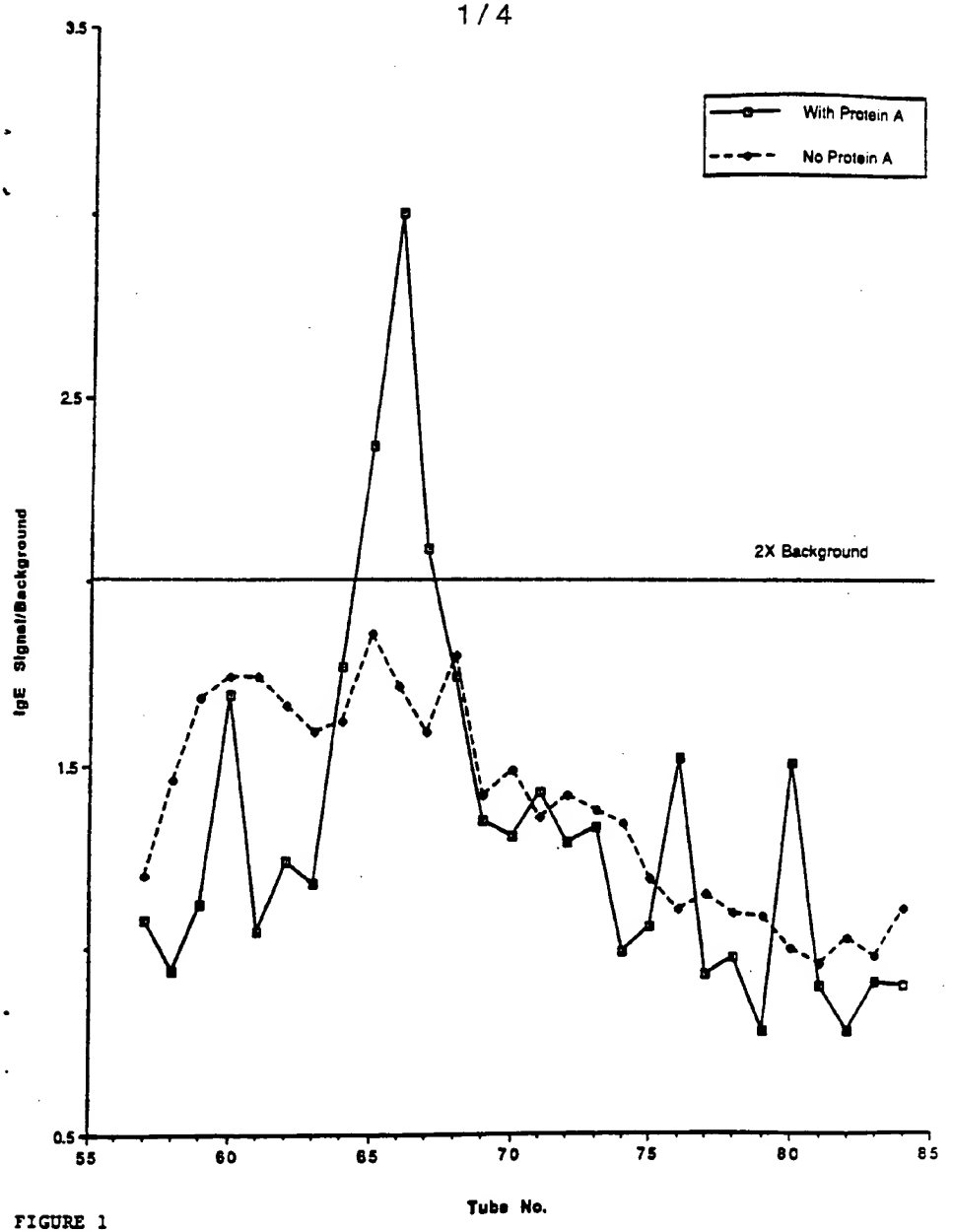
23. A diagnostic kit according to claim 22, wherein the protein allergen is affixed to a solid substrate.

24. A composition comprised of a structural analog of an epitope of an H. pylori allergen, wherein the structural analog binds to an IgE paratope.

25. The composition of claim 24, wherein the structural analog is an anti-idiotype antibody.

26. A composition comprised of a purified polyclonal antibody directed to a polypeptide allergen of H. pylori.

27. A composition comprised of a monoclonal antibody directed to a polypeptide allergen of H. pylori.


With Protein A
No Protein A
2X Background
IgE Signal/Background
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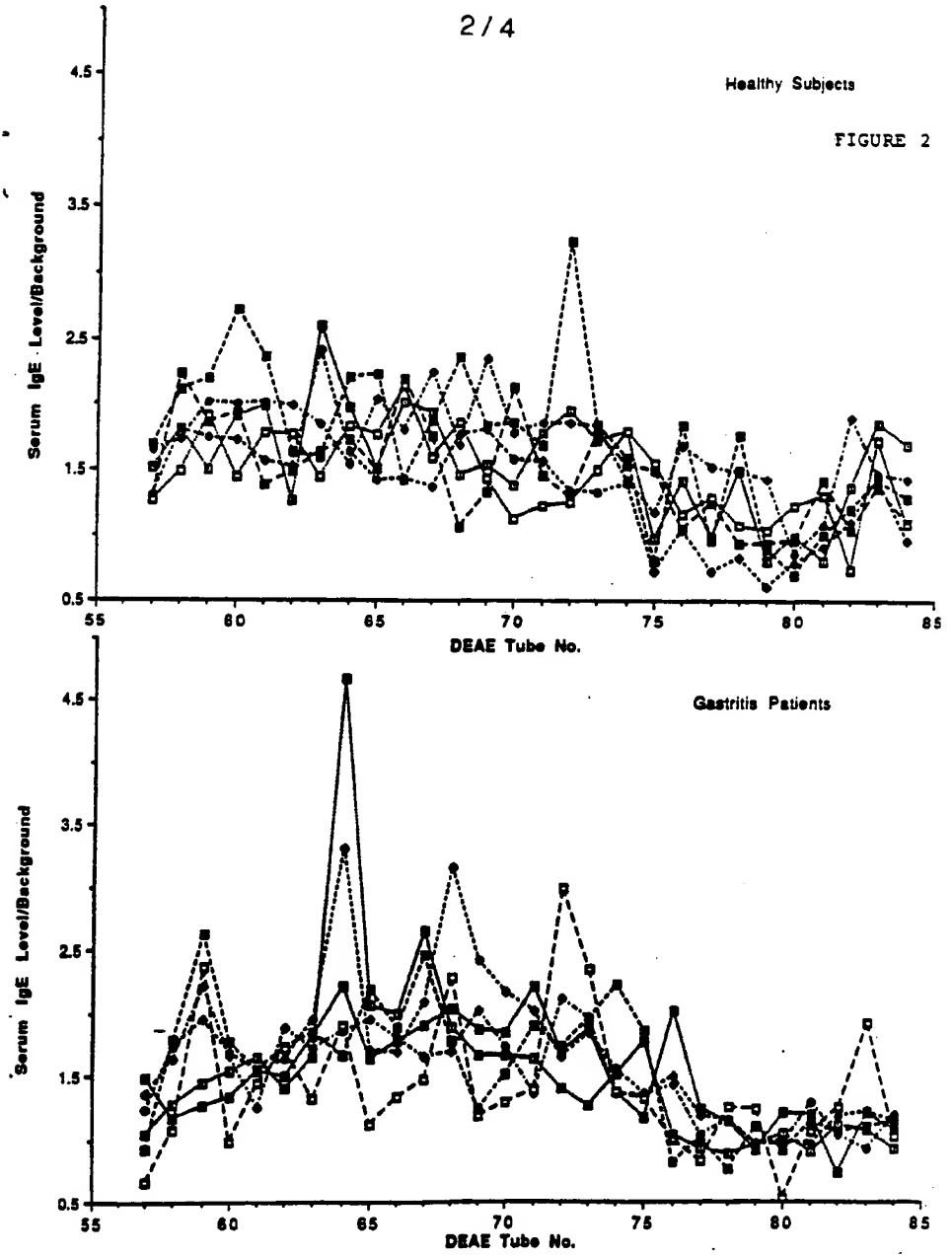
2.5
1.5
0.5
55
60
65
70
75
80
85
Tube No.

FIGURE 1


Healthy Subjects
Serum IgE Level/Background
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3.5
2.5
1.5
0.5
55
60
65
70
75
80
85
DEAE Tube No.
Gastritis Patients
Serum IgE Level/Background
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3.5
2.5
1.5
0.5
55
60
65
70
75
80
85
DEAE Tube No.

FIGURE 2

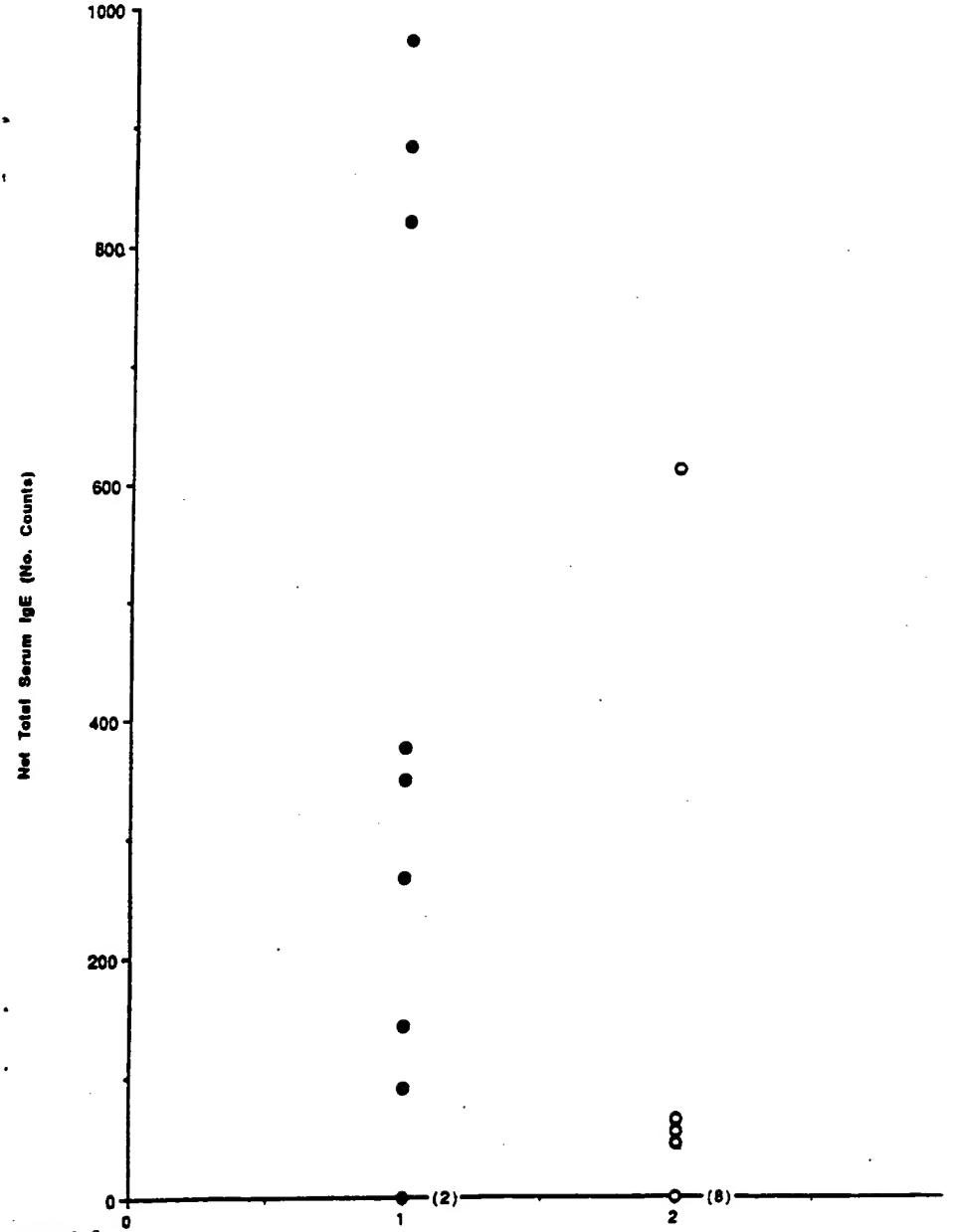

FIGURE 3

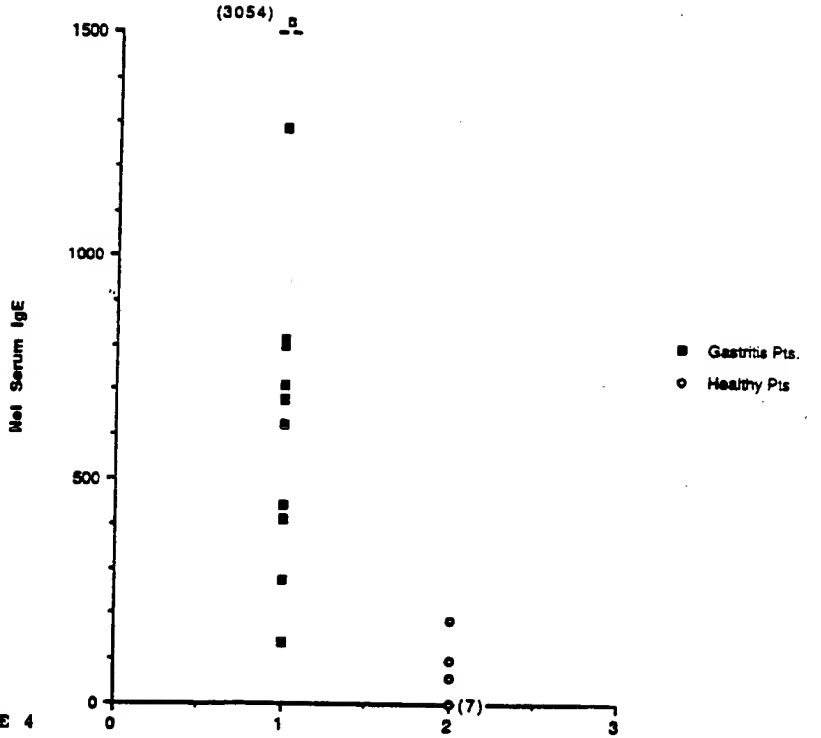
(3054)
1500
1000
500
0
Nel Serum IgE
(7)
0
1
2
3
Gastritis Pts.
Healthy Pts

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03284

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/569; A61K 39/106, 39/35
US CL :435/7.32; 436/513; 424/92; 530/868
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.32, 962, 965, 975; 436/513, 518, 547, 548; 424/92; 530/868, 388.4, 389.5, 412, 416, 422

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG search terms:helicobacter pylori or campylobacter pylori, IgE, IgG or IgA interference, bacterial allergens, protein A, author name search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,870,053 (Zalisz et al) 26 September 1989, column 4, lines 30-54.	11
Y,P	Gastroenterology, Volume 101, No. 1, issued July 1991, Aceti et al, "Basophil-Bound and Serum Immunoglobulin E Directed Against Helicobacteri pylori in Patients With Chronic Gastritis", pages 131-137, expecially abstract on page 131, page 133, left column, second full paragraph and page 136, left column first full paragraph.	1-27
A,P	The New England Journal of Medicine, Volume 324, No. 15, issued 11 April 1991, Peterson, "Helicobacter Pylori and Peptic Ulcer Disease", pages 1043-1048.	1-27
Y	Journal of Immunology, Volume 126, No. 2, issued February 1981, Gleich et al, "Measurement of IgG Blocking Antibodies By Interference in The Radioallergosorbent Test", pages 575-579, abstract on page 575 and page 578, lines 33-34.	1-4, 12-14
A	US, A, 4,849,337 (Calenoff et al) 18 July 1989.	1-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 28 July 1992	Date of mailing of the international search report 05 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CAROL BIDWELL Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 3,983,008 (Shinozaki et al) 28 September 1976, column 1, lines 55-57, column 2, lines 22-25.	5
Y	EP, A, 282,018 (Hoechst Aktiengesellschaft) 14 September 1988, see English abstract.	5-6
A,P	EP, A, 451,800 (Abbott Laboratories) 16 October 1991	1-10, 12-18, 22-23
A	D.M. Weir, "Handbook of Experimental Immunology", published 1978 by Blackwell Scientific Publications (Oxford), pages 2.1-2.17.	5-6
Y	Trends in Biotechnology, Volume 3, No. 7, issued 1985, Scott, "Monoclonal antibodies-approaching adolescence in diagnostic immunoassays", pages 170-175.	26-27